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Creatin(in)e and Maillard Reaction Products as Precursors of Mutagenic Compounds: Effects of Various Amino Acids

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ABSTRACT

The participation of creatin(in)e and Maillard reaction products in developing mutagenic activity was studied in model systems. Glucose and an amino acid were boiled under reflux for 2 h at 130°C together with creatine or creatinine dissolved in water–diethylene glycol (1:6). Threonine produced the highest mutagenic activity, 1068 revertants per μmol amino acid, towards TA98 after S9 activation, followed by glycine (410 rev/μmol) and lysine (246 rev/μmol). Proline, glutamic acid and the sulfur-containing amino acids produced less than 40 rev/μmol. Protein-bound amino acids produced no detectable mutagenic activity. When added to the reaction mixtures, the pure Maillard reaction products increased the mutagenic activity significantly. All precursors used occur in free form in meat. Work is now in progress to identify the mutagenic compounds produced in the model systems and to establish whether they also occur in fried meat.

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INTRODUCTION

By use of the Ames test, mutagenic activity has been found to develop in beef and fish during frying or boiling (Sugimura & Nagao, 1979; Weisburger & Spingarn, 1979; Bjeldanes *et al.*, 1982). Heat-processed foods of vegetable origin such as cereals, potatoes and bakery products seem to be less active (Pariza *et al.*, 1979; Spingarn *et al.*, 1980*a*).

Hitherto, two main groups of mutagens occurring in fried meat or fish have been identified. One group consists of pyrolysis products of amino acids, where especially 'Trp-P-1', 'Trp-P-2' and 'Glu-P-2' have been shown to be very active in the Ames test and also to be hepatocarcinogens (Sugimura & Nagao, 1979; Sugimura *et al.*, 1982). The other group of mutagens are the so-called IQ compounds ('IQ', 'MeIQ' and 'MeIQx'; Kasai *et al.*, 1980; Spingarn *et al.*, 1980*b*). The IQ compounds are the most potent mutagens so far analysed in the Ames test and their tumour-inducing effects are now being studied through an *in vivo* long-term animal test by the Sugimura group. According to Spingarn *et al.* (1980*b*) and Kasai *et al.* (1981), the IQ-compounds constitute the main part of the mutagenic activity demonstrated in fried meat.

The precursors of the IQ compounds are still unknown, but a possible route for their formation has recently been given, suggesting creatinine and Maillard reaction products to be the precursors (Jägerstad *et al.*, 1982). The 'Maillard reaction' means the complex reaction between reducing sugars, such as glucose, and amines, including amino acids (Mauron, 1981). The participation of the Maillard reaction in producing mutagenic activity in fried beef has been proposed by several authors (Spingarn & Garvie, 1979; Spingarn *et al.*, 1980*a*; Sugimura *et al.*, 1982; Shibamoto, 1982), although the mechanism for its participation is not yet established.

The present study demonstrates, by means of model systems, that creatine or creatinine, glucose and certain amino acids produce high mutagenic activity when heated under conditions realistic for meat frying. All precursors occur naturally in meat. The results are discussed in relation to other reports based on similar model systems.

Abbreviations:
Trp-P-1 3-amino-1,4-dimethyl-5H-pyrido [4,3-b]-indole; *Trp-P-2* 3-amino-1-methyl-5H-pyrido [4,3-b]-indole; *Glu-P-2* 2-aminodipyrido [1,2-a:3',2'-d] imidazole; *IQ* 2-amino-3-methyl-imidazo [4,5-f] quinoline; *MeIQ* 2-amino-3,4-dimethylimidazo [4,5-f] quinoline; *MeIQx* 2-amino-3,8-dimethylimidazo [4,5-f] quinoxaline.

MATERIALS AND METHODS

Model experiments

Creatine (10 mmol), D-glucose (5·0 mmol) and an amino acid (10 mmol; see Table 1) were dissolved in water (10 ml) and diethylene glycol (DEG, 50 ml). In some experiments, an equimolar amount of creatinine was used instead of creatine. Model mixtures excluding one reactant at a time were

TABLE 1

Mutagenic Activities in Ames Test towards TA98 in the Presence of S9 Mix after Refluxing a Solution of Creatine (10 mmol), D-Glucose (5·0 mmol) and an Amino Acid (10 mmol) or Albumin (1·0 g) in Water (10 ml) and Diethylene Glycol (50 ml) for 2 h

Amino compound	*Temperature* (°C)[a]	*Final pH*[b]	*Rev/μmol amino acid*	*Rev/plate*[c] *for sample of* 10 μl	20 μl	50 μl
L-Threonine	132	6·9	1 068 ± 281	140 ± 26	422 ± 66	—
Glycine	129	6·6	410 ± 59	71 ± 11	131 ± 13	—
L-Lysine	130	6·1	246 ± 108	45 ± 15	75 ± 42	—
L-Alanine	130	6·9	199 ± 32	31 ± 7	64 ± 4	—
L-Serine	130	6·9	197 ± 85	35 ± 17	63 ± 23	—
L-Leucine	131	7·2	161 ± 22	26 ± 4	55 ± 5	—
L-Histidine	130	7·0	126 ± 33	21 ± 7	41 ± 7	—
L-Arginine[d]	130	6·2	101 ± 29	18 ± 5	32 ± 10	—
L-Valine	130	6·9	91 ± 29	14 ± 5	33 ± 9	59 ± 8
L-Isoleucine	130	6·8	75 ± 26	14 ± 4	22 ± 8	—
L-Asparagine	127	6·7	63 ± 23	—	22 ± 5	64 ± 13
L-Tyrosine	126	5·8	56 ± 23	—	19 ± 9	45 ± 17
L-Aspartic acid	129	6·6	55 ± 17	—	19 ± 8	40 ± 15
L-Phenylalanine	131	6·7	50 ± 26	17 ± 6	23 ± 8	26 ± 4
L-Tryptophan	132	6·8	50 ± 27	12 ± 10	17 ± 9	19 ± 10
L-Cysteine[d]	128	6·7	40 ± 13	—	12 ± 7	20 ± 13
L-Methionine	128	7·4	34 ± 17	—	14 ± 5	14 ± 5
L-Glutamine	133	5·6	33 ± 12	—	13 ± 4	27 ± 3
L-Proline	131	5·1	31 ± 22	—	9 ± 6	18 ± 3
L-Glutamic acid	128	7·1	30 ± 13	—	10 ± 7	27 ± 3
L-Cystine	128	6·4	19 ± 8	—	7 ± 3	13 ± 2
Albumin	124	6·5	0	0	0	0

[a] Mean of four observations at 30-min intervals.
[b] After cooling; mean of two experiments.
[c] Mean and SD of twelve observations after subtraction of spontaneous revertants (20).
[d] As monohydrochloride.

included as controls. In one experiment, a 20% (w/v) aqueous solution (5 ml) of solubilized albumin was substituted for the amino acid and 5 ml of the water. All mixtures were boiled under reflux for 2 h, during which the temperature was monitored at 30-min intervals. The pH was measured after cooling. All experiments were performed in duplicate.

The influence of the Maillard reaction was further studied by adding pure 2-methylpyridine (0·50 ml, 5·4 mmol) or 2,5-dimethylpyrazine (0·50 ml, 4·6 mmol; freshly distilled) to model mixtures containing creatinine (10 mmol), D-glucose (5 mmol) and either L-alanine (10 mmol) or glycine (10 mmol) in water (10 ml) and DEG (50 ml; see Table 2). These mixtures were boiled under reflux as described above. In one experiment the water content was reduced from 10 to 3 ml.

TABLE 2
Effect of Adding a Pure Maillard Reaction Product (5 mmol) in Experiments Listed in Table 1 (Creatinine Used Instead of Creatine)

Amino compound	*Added product*	*Rev/μmol amino acid*	*Rev/plate for sample of*		
			10 μl	*20 μl*	*50 μl*
Glycine	—	460 ± 59	75 ± 11	164 ± 12	350 ± 65
Glycine	2-Methylpyridine	689 ± 137[a]	134 ± 16	234 ± 44	472 ± 58
Glycine	2,5-Dimethylpyridine	688 ± 154[a]	133 ± 32	242 ± 11	452 ± 54
L-Alanine	—	354 ± 64	60 ± 17	105 ± 10	325 ± 17
L-Alanine	2-Methylpyridine	642 ± 93[a]	118 ± 21	232 ± 15	460 ± 30

[a] $p < 0{\cdot}001$ (compared with no added Maillard product).

Mutagenicity test

The TA 98 strain of *Salmonella typhimurium* was used. Overnight cultures of bacteria prepared according to Ames *et al.* (1975) in nutrient broth (Antibiotic Medium No. 2, Oxoid) were used for mutation assay. Samples diluted ten times with water were assayed in amounts of 10, 20 μl and/or 50 μl per plate. Triplicates of samples were mixed in tubes with 0·5 ml of a mix of 2·5% liver homogenate supernatant (S9) prepared from rats treated with Arochlor 1254 brand polychlorinated biphenyls as described by Ames *et al.* (1975). The optimum amount of liver homogenate added to the S9 mix was examined for the model mixture containing glycine as amino acid (see above), as well as for the positive

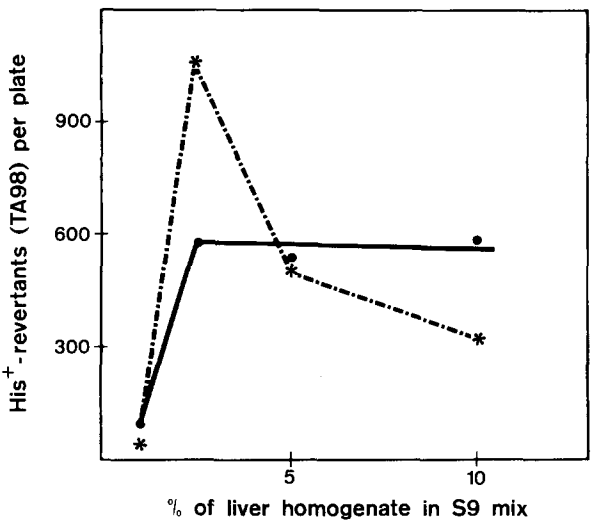

Fig. 1. The effect of various amounts of liver homogenate in S9 mix on the mutagenicity of 2-amino-anthracene (∗ – · – ∗, 1 μg/plate) and a model mixture (· —— ·) towards TA98. The model mixture contained creatine (10 mmol), D-glucose (5 mmol) and glycine (10 mmol) dissolved in 10 ml of water and 50 ml of diethyleneglycol. Mean of three observations per plate.

control, 2-amino-anthracene (2-AA) (see Fig. 1). Samples without S9 mix were diluted with 0·5 ml of a sodium phosphate buffer (167 mM, pH 7·4), in order to get equal volumes. All tubes were pre-incubated for 20 min in a water-bath kept at 37 °C. At the end of the pre-incubation period, 0·1 ml of the bacterial culture was added to each tube and, after mixing with 2 ml of melted topagar, poured into Petri dishes of minimal agar medium. Blanks and positive controls, usually 2-AA (1 μg/plate), were always included. $^{+}$His-revertants were counted after incubation at 37 °C for 48 h. The values were corrected for spontaneous revertants (20 ± 4 per plate). The coefficient of variation for six different analyses of 2-AA was 22 % for TA 98 after S9 activation.

RESULTS AND DISCUSSION

Mutagenicity in the model experiments

Initial experiments reported earlier (Jägerstad *et al.*, 1982) showed a considerable production of mutagenic activity when creatinine was

heated with typical Maillard reactants, i.e. glucose and an amino acid (glycine or alanine). Important for producing mutagenic activity was the high boiling point (*ca.* 130°C) of the solvent, which was a 1:6(v/v) mixture of water and diethyleneglycol. Boiling all reactants in water only produced no mutagenic activity. Also essential for the production of mutagenicity was the combination of all three reactants. Combining any two of these reactants produced no mutagenic activity.

As reported earlier (Jägerstad *et al.*, 1982), the mutagens produced in the model experiments required S9 activation, and the mutagenic activity was considerably higher towards TA98 than towards TA100. The optimum concentration of liver homogenate in the S9 mix is demonstrated in Fig. 1. There is a sharp peak at 2·5% of liver homogenate for the positive control, 2-amino-anthracene. In the model mixture, too, the maximum activity is reached at 2·5% and is unaffected by higher concentrations of liver homogenate.

Effect of creatin(in)e

Only foods of animal origin such as meat and fish contain creatine, which is converted to creatinine to a large extent when heated (Hughes, 1960; Sulser, 1978). When heated for 2 h with glucose and glycine in refluxing water—DEG (1:6), creatine produced 410 ± 59, and creatinine, 460 ± 59 rev/μmol glycine ($p > 0{\cdot}05$). On the other hand, in the presence of alanine instead of glycine, creatine produced 199 ± 32, and creatinine, 354 ± 64 rev/μmol alanine, which differed significantly ($p < 0{\cdot}001$). Thus, creatinine was a more efficient reactant compared with creatine.

Effects of other amino acids

The results for different amino acids are shown in Table 1. Mean values of the boiling temperature and the final pH after cooling are indicated. Although generally between 6 and 7, the final pH varied slightly according to the amino acid used and may have affected the resulting mutagenicity.

As seen from Table 1, threonine produced the highest activity, followed by glycine and lysine. The lowest mutagenic activities (<40 rev/μmol) were obtained for the sulfur-containing amino acids as well as for proline and glutamic acid. Protein-bound amino acids (albumin) produced no activity. The number of revertants per plate is also given in Table 1 for different sample sizes (10, 20 and 50 μl, diluted 1:10). These values generally show a good dose response. On the other hand, the significance

of the mutagenic activity might be questioned when around, or less than, 20 revertants per plate were found after subtraction of spontaneous revertants. This applies to the amino acids listed below L-aspartic acid in Table 1.

A Japanese group (Yoshida & Fukuhara, 1982) has studied the same precursors as used in our model system, but combined them in other ways and under other conditions. They heated equal amounts of creatine and an amino acid *without* glucose or solvent for 1 h at 200 °C. The sulfur-containing and aromatic amino acids were among the most active. Cystine produced the highest activity, 655 rev/μmol, followed by threonine (135), phenylalanine (112) and methionine (107 rev/μmol). This ranking order of the amino acids is totally different from that found in the present study.

Pyrolysis of pure amino acids also produces mutagenic activity in the Ames test. Such a study has been presented by Nagao *et al.* (1977). The activities observed in their system led to still another quite different ranking order of the amino acids, tryptophan, serine and glutamic acid being the most active.

Generally, there are several difficulties in comparing the capacities of different reactants to produce mutagenic activity in the Ames test. The reaction velocity may differ according to the reaction mixtures and/or heating conditions. Furthermore, different reactants may give rise to varying amounts of products, which might interfere with the S9 activation.

Maillard reaction and mutagenicity

As can be seen from Table 2, addition of the Maillard reaction products, 2-methylpyridine and 2,5-dimethylpyrazine, to the model mixtures containing either alanine or glycine as amino acid increased the mutagenic activity by between 50 and 80 %—a significant increase ($p < 0{\cdot}001$). The influence of the Maillard reaction in producing mutagenicity was further supported by meat experiments. The mutagenic activity was found to increase with the glucose content of raw beef and also with the development of brown colour in the meat crust after frying (Jägerstad *et al.*, 1982).

The rôle of the non-enzymatic browning reaction (Maillard reaction) in producing mutagenic activity has been studied in model systems by several investigators (Spingarn & Garvie, 1979; Yoshida & Okamoto, 1980*a*, *b*; Aeschbacher *et al.*, 1981). However, when dilute aqueous

solutions of reducing sugars and amines, including amino acids, are refluxed, the temperature remains at 100 °C. The mutagenic activities developed in such systems towards TA98 after S9 activation were < 1 % of those found in the present study, where a reaction temperature of *ca.* 130 °C was used. High temperatures and low water content are known to favour the Maillard reaction (Mauron, 1981). When the water content in our model system was reduced from 10 to 3 ml, the reaction temperature rose to *ca.* 150 °C, and the mutagenic activity increased by 130 % ($p < 0{\cdot}001$). In a model system used by Yoshida & Okamoto (1980*c*), equal amounts of glucose and creatine were heated for 2 h in sealed tubes. No mutagenic activity was produced below 125 °C. At 150–250 °C, however, detectable mutagenicity was produced with an optimum value corresponding to 24 rev/μmol creatine at 150 °C.

Precursors occurring in raw beef

The precursors responsible for mutagenic activity in the present study occur in raw beef in the free form. Raw beef normally contains 0·1 % of glucose and 0·2 % of glucose-6-phosphate (Laser Reuterswärd *et al.*, 1981). The creatine content of raw beef amounts to around 0·4 %, half of which is converted to creatinine during frying (Jägerstad *et al.*, 1982). The amino acids do not necessarily arise through protein hydrolysis or pyrolysis during cooking. The total amount of free amino acids is 0·1–0·3 % in raw beef. Up to half of this is alanine, glutamic acid and taurine, each at a level of 0·01–0·05 % (Sulser, 1978). The amino acids thus normally seem to be the limiting factor in the possible reaction between glucose, amino acids and creatine in beef. In the model mixtures, glucose, amino acids and creatine were used in concentrations between 1 and 2 %, which is about ten times higher than the natural concentrations of these compounds in raw beef. Supposing that glycine constitutes 0·01 % of raw beef; this should correspond to about 1 μmol of glycine per gram of beef and thus a theoretical production of about 400 rev/gram of meat crust. This figure is in agreement with the mutagenic activity produced in beef during roasting (to be published).

Identity of the mutagens

Hitherto, no information is available regarding the identity of the mutagens produced in the model systems referred to in the present study.

Work is in progress to identify these mutagens. We are primarily looking for the IQ compounds to support an earlier suggested reaction mechanism (Jägerstad *et al.*, 1982) but other compounds may be formed as well. Regardless of whether the mutagenic activity produced in the model system appears to be due to already known mutagenic compounds or hitherto unknown compounds, these substances have to be further evaluated using other test systems in order to clarify their tumour producing capacity. However, identification of precursors of such compounds might give opportunities to affect their formation during frying or heating of foods.

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